Macrophages in asthma

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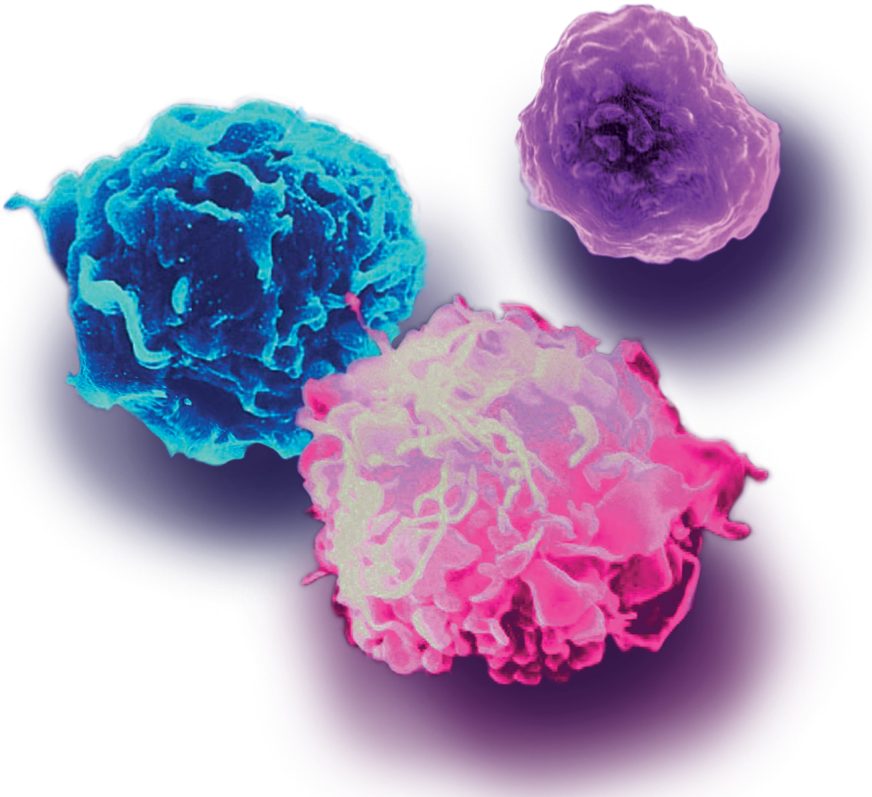
Chapter 7



PGE2-treated macrophages inhibit development of allergic lung inflammation in mice

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Published in Journal of Leukocyte Biology, 2016

Abstract

In healthy lungs many macrophages are characterized by interleukin(IL)10 production and few are characterized by expression of interferon regulatory factor 5 (IRF5) (formerly M1) or YM1 and/or CD206 (formerly M2), while in asthma this balance shifts towards few producing IL10 and many expressing IRF5 or YM1/CD206. In this study we tested whether redressing the balance by reinstating IL10 production could prevent house dust mite (HDM)-induced allergic lung inflammation.

Prostaglandin E2 (PGE2) was found to be the best inducer of IL10 in macrophages *in vitro*. Mice were then sensitized and challenged to HDM during a 2-week protocol while treated with PGE2 in different ways. Lung inflammation was assessed 3 days after the last HDM challenge.

HDM-exposed mice treated with free PGE2 had fewer infiltrating eosinophils in lungs and lower YM1 serum levels than vehicle-treated mice. Macrophage-specific delivery of PGE2 did not affect lung inflammation. Adoptive transfer of PGE2-treated macrophages led to fewer infiltrating eosinophils, macrophages, (activated) CD4+ and regulatory T lymphocytes in lungs.

Our study shows that redirecting macrophage polarization by using PGE2 inhibits development of allergic lung inflammation. This beneficial effect of macrophage repolarization is a novel avenue to explore for therapeutic purposes.

Introduction

Allergic asthma is a prevalent disease of the airways characterized by chronic inflammation of the airways with infiltration of eosinophils, Th2 lymphocytes and the presence of alternatively activated macrophages [1–6]. The role of macrophages in the development and progression of asthma has been a hot topic of late. Alveolar macrophages were shown to be derived from embryonic progenitors, as opposed to circulating monocytes (and thus hematopoietic stem cells), and were shown to self-maintain during steady-state conditions [7–9]. This led to studies investigating the origin of macrophages during allergic inflammation that characterizes asthma [10, 11]. These studies indicate that immediately after allergen exposure, monocytes are recruited to the lung and may develop into alveolar macrophages. At later time points though, resident alveolar macrophages proliferate and constitute the main component of the alveolar macrophage pool. In addition, some recent and older studies have also highlighted the importance of resident alveolar macrophages in the maintaining homeostasis in lung tissue and immigrating monocyte-derived macrophages in contributing to allergic inflammation [10–13]. The picture that arises is one of fast recruitment of monocytes after allergen exposure to fight the perceived dangers of the allergen with consequently inflammation and subsequent expansion of alveolar macrophages to restore homeostasis. In asthma, however, this homeostasis is not achieved.

For yet unknown reasons the Th2-type inflammation persists giving rise to eosinophil infiltration and switching of macrophages to an alternatively activated phenotype by the high presence of cytokines like IL4 and IL13 [14]. Macrophages can be polarized into many different phenotypes that are hard to define *in vivo* [15, 16]. We have previously shown that many macrophages in healthy lungs are characterized by production of IL10 and few are characterized by expression of IRF5 (classically activated macrophages or formerly known as M1) or YM1 and/or CD206 (alternatively activated macrophages or formerly known as M2), while in asthma this balance shifts towards few producing IL10 and many expressing IRF5 or YM1/CD206 [1, 4, 5, 17]. This finding suggested to us it would perhaps be possible to re-instate homeostatic behavior of macrophages by inducing IL10 production in macrophages *in vivo* in lung tissue and thereby treat allergic lung inflammation. In order to do this we first investigated what stimulus could effectively induce IL10 production in macrophages and this proved to be PGE2. We then continued with several ways of offering PGE2 to lung macrophages in mice to study its effect on the development of house dust mite (HDM)-induced allergic lung inflammation, i.e. intranasal treatment with free PGE2, intranasal treatment with PGE2 coupled to mannosylated albumin for macrophage-specific uptake,

and intratracheal adoptive transfer of PGE2-treated macrophages. In addition, we also studied whether inducing homeostatic behavior in hematopoietic stem cell-derived macrophages differed from resident lung macrophages with respect to their effects on development of allergic lung inflammation to investigate if macrophage origin could influence their homeostatic behavior.

Materials and methods

Animals

Female BALB/cOlaHsd mice aged 6-8 weeks were purchased from Harlan (Horst, The Netherlands). DO11.10 T cell TCR-transgenic mice were bred and maintained at the Central Animal Facility of the University of Groningen. The mice were housed in groups of 4 and had *ad libitum* access to water and food. The Institutional Animal Care and Use Committee of the University of Groningen approved these experiments (application numbers 6272A, 6272B, 6272C, and 6272F), which were performed under strict governmental and international guidelines.

Macrophage cell line RAW264.7 cultures

Murine RAW264.7 macrophages (passage 3, ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagles Medium (DMEM + pyruvate, Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum (FCS, Invitrogen, Bleiswijk, the Netherlands), 4,5 g/L glucose (Biowhittaker BE12-604F), 2 mM L-glutamine (Gibco®, Life Technologies, Bleiswijk, the Netherlands), and 11 mg/l gentamicine (Gibco®). Cells were used between passage 5 and 15.

Synthesis of mannosylated human serum albumin (HSA)

52.7 µmol (14.3 mg) p-aminophenyl-α-D-mannopyranoside (Sigma) was dissolved in 4 ml 80% ethanol and was allowed to react with 50 µl thiophosgene (Sigma) for 1.5 hours at room temperature, followed by 2.5 hours under nitrogen atmosphere. After this, 4 ml H_20 was added and the pH was set to 6 using 1 N NaOH. Subsequently, the solvent was removed using a rotary evaporator (p=0 mbar, T=60˚C). The residue was dissolved in 3 ml 0.1 M sodium carbonate buffer pH 9.0 and added slowly to 2.1 µmol (0.14 g) HSA (Sanquin, Amsterdam, the Netherlands; freeze-dried after being dialyzed against ultrapure water 4 times) in 12 ml of the same buffer. The mixture was allowed to stir for 18 hours at room temperature.

The mixture was dialyzed against PBS using 30 kDa Amicon centrifuge tubes (Millipore, Amsterdam, the Netherlands) for 20 min at 8˚C and 3000 rpm. The residue was

resuspended in PBS and dialyzed again twice. The subsequent residue was purified by size-exclusion chromatography using a Superdex 200 column on an Äkta System (GE Healthcare, Uppsala, Sweden) to isolate the monomeric form of the mannosylated protein. The eluate was extensively dialyzed against ultrapure water. The final product was freeze-dried overnight for long-term storage at -20˚C and was identified by matrix-assisted laser desorption/ionisation – time-of-flight (MALDI-TOF) analysis.

Synthesis of PGE2-Mannose-HSA

17 µmol (6 mg) PGE2 (Cayman Chemical Company) was dissolved in 400 µl dimethylformamide (DMF, Sigma). Additionally, 21 µmol (4.32 mg) dicyclohexylcarbodiimide (DCC; Sigma) and 21 µmol (2.4 mg) N-hydroxysuccinimide (NHS; Pierce, Rockford, Illinois) were dissolved separately in DMF at a concentration of 10 mg/ml. First, the DCC solution was added to the PGE2 solution slowly. Then, the NHS solution was added drop-wise and the solution was mixed after each drop. The mixture was additionally mixed for another 45 minutes.

0.36 µmol (25.4 mg) mannosylated HSA was dissolved in PBS at the concentration of 10 mg/ml. The PGE2 reaction mixture was added to the mannosylated HSA solution slowly and was allowed to react overnight at room temperature.

The reaction product was dialyzed extensively against PBS using a dialyzing membrane (ThermoScientific Slide-A-Lyzer, 10 kDa). The subsequent reaction product was purified by size-exclusion chromatography using a Superdex 200 column as described for mannosylated HSA. The eluate was extensively dialyzed against PBS using 30 kDa Amicon centrifuge tubes. The reaction product was identified by MALDI-TOF analysis.

MALDI-TOF analysis of mannosylated HSA and PGE2 coupled to mannosylated HSA

Mannosylated HSA and PGE_2-mannose-HSA were characterized by MALDI-TOF (Voyager-DE PRO, Applied Biosystems, Bleiswijk, the Netherlands). The samples were analyzed in a concentration of 10 mg/ml in a mixture of 2 mg 3,5-dimethoxy-4-hydroxycinnamic acid (SA) / 0.1% trifluoroacetic acid / 50% acetonitrile. Bovine serum albumin and unmodified HSA were used as internal standards. The number of mannose residues coupled was calculated by subtracting the mass of HSA from the mass of the mannosylated HSA and dividing this number by the mass of the mannose residue plus its linker molecule. The number of PGE2 residues coupled was calculated similarly using the mass of the mannosylated HSA to subtract from the PGE2 conjugate.

Induction and analysis of IL10

For IL10 induction, RAW264.7 macrophages were plated in 12 wells plates with 50.000 cells per well and stimulated for 48 h with 10 ng/ml LPS (Sigma Aldrich), 10 µM PGE2 (Cayman Chemical Company), or 100 µM adenosine (Sigma). During the last 16 hours, 3 µg/ml brefeldin (eBioscience, San Diego, CA) was added to prevent release of IL10 from cells. After 48 hours of incubation, cells were detached from the wells by incubating in phosphate-buffered saline (PBS) with 4 mg/ml lidocaine HCl (Sigma), 10 mM ETDA (Sigma) and 10% FCS at room temperature for 15 min. Cell were washed twice with PBS with 2% FCS and 5 mM EDTA (PFE) at 4 °C and subsequently fixed and permeabilized at 4 °C for intracellular IL10 staining using a fix/perm kit from eBioscience. After washing with permeabilization buffer, cells were incubated in the dark at 4 °C for 30 min with phycoerythrin (PE)-labeled anti-murine IL10 (eBioscience) diluted 1:100 in PFE with 2% normal mouse serum. After washing with permeabilization buffer, cells were resuspended in PFE and subsequently analyzed using a BD Facsarray (BD Biosciences, Breda, The Netherlands). Data were analyzed using FlowJo Software (Tree Star, Ashland, USA). A Fluorescent Minus One (FMO) control was included for proper gating during data analyses.

In vitro testing of bioactivity of PGE2 coupled to mannosylated HSA

RAW 264.7 macrophages (25,000 cells/well) were plated in 24 wells plates and stimulated with 100 µg/ml, 10 µg/ml and 1 µg/ml $PGE2_{11}$-$mannose_{10}$-HSA or free PGE2 in equimolar concentrations (72 nM, 7,2 nM and 0,72 nM) for 48 hours. As controls, 100 µg/ml, 10 µg/ml and 1 µg/ml $mannose_{10}$-HSA or HSA were added to the cells. After 48 hours of incubation flow cytometric analysis of IL10 expression was performed as described for the induction of IL10 expression above.

Effects of PGE2 and $PGE2_{11}$-$mannose_{10}$-HSA in a murine model of asthma

Female Balb/c Mice (n=4 per group) were exposed intranasally to whole body house dust mite (HDM) extract (Dermatophagoides pteronyssinus, Greer laboratories, Lenoir, USA) according to a 14-day model [4]. Briefly, mice were anesthetized with isoflurane and received a sensitization dose of 100 µg HDM in 40 µl PBS on day 0 and were challenged with 10 µg HDM in 40 µl on day 7-11 to induce allergic lung inflammation. Mice exposed to 40 µl PBS according to the HDM protocol served as healthy controls (n=8).

The mice were treated on day 7, 9 and 11 with either $PGE2_{(11)}$-$mannose_{10}$-HSA in three different concentrations (1,85 mg/kg, 3,70 mg/kg and 7,40 mg/kg), or with free PGE2 in the same molar concentrations (0,10 mg/kg, 0,20 mg/kg and 0,40 mg/kg). As a control, one group received mannose-HSA without PGE2 coupled to it. They were

given the same molar concentration mannose-HSA as in the highest PGE2-mannose-HSA treated group (7,0 mg/kg mannose-HSA). Additionally, as a control, one group was treated with PBS. All treatments were administered intranasally in a volume of 37 µl directly after the HDM challenge of that day. Mice were sacrificed on day 14 of the protocol. A flow chart of the experiment is depicted in figure 1A.

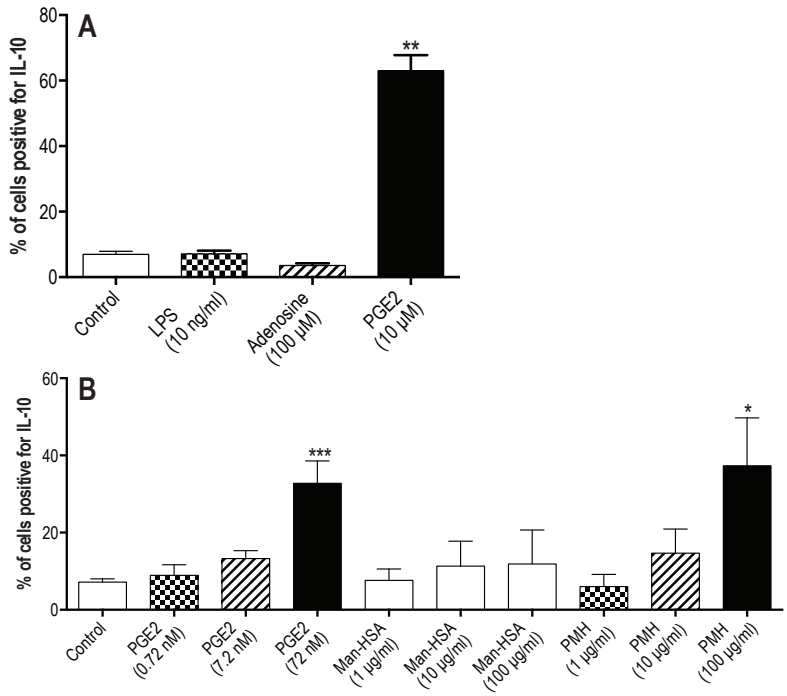

Figure 1A: The percentage of RAW 264.7 macrophages expressing IL10 is significantly higher after being stimulated with 10 µM PGE2 as compared to nonstimulated controls (n=8). Stimulation with 10 ng/ml lipopolysaccharide (LPS) or 100 µM adenosine did not result in more macrophages expressing IL-10 (n=3). **P<0.01 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing the different treatment groups vs. control.

Figure 1B: The percentage of RAW 264.7 macrophages expressing IL10 is significantly higher after being stimulated with increasing amounts PGE2 or with equimolar increasing amounts of PGE2 coupled to mannosylated human serum albumin (PMH) as compared to nonstimulated controls (n=7). Equimolar amounts of mannosylated human serum albumin (Man-HSA) did not affect the number of IL10-expressing macrophages (n=7). *P<0.05, ***p<0.001 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing the different treatment groups vs. control.

The left lung lobe was collected to isolate lung cells from digested lung for flow cytometry and the right lung was inflated with 0.5 mL 50% Tissue-Tek O.C.T. compound (Sakura, Finetek Europe B.V., Zoeterwoude, The Netherlands) in PBS and zinc-fixed for histological analyses.

Effects of adoptive transfer of PGE2-treated lung and bone-marrow-derived macrophages in a murine model of asthma

To investigate the effects of adoptive transfer of PGE2-treated embryonic/lung-derived versus hemopoietic macrophages on development of allergic lung inflammation, mice were exposed to HDM in combination with adoptive transfer of macrophages. Female Balb/c mice (n=4 per group) were sensitized to HDM intranasally with 100 µg whole body HDM extract in 40 µl PBS on day 0. Control mice received only PBS. On day 6, control mice received 50 µl PBS intratracheally and HDM-exposed mice received PBS, 0.15 10^6 unstimulated, or 0.15 10^6 PGE2-treated alveolar or bone-marrow-derived macrophages in 50 µl of PBS intratracheally. On day 7-11, control mice were subsequently challenged intranasally with 40 µl PBS and the other groups were challenged with 10 µg HDM in 40 µl PBS to induce allergic lung inflammation. Mice were sacrificed on day 14 and the left lung was collected for flow cytometry. The right lung was inflated with 50% Tissue-Tek© O.C.T.Ô compound (Sakura, Finetek Europe B.V., Zoeterwoude, The Netherlands) in PBS and fixed in zinc for histology. A flow chart of the experiment is depicted in figure 1B.

Culture of PGE2-treated bone marrow-derived and lung macrophage subsets

Bone marrow-derived macrophages: These were cultured from bone marrow of control mice with 10 ng/ml M-CSF (Peprotech, Hamburg, Germany) as described by us before [3]. In short, bones of hind legs were removed and placed in petri dish with PBS. The ends of the femur and tibia were cut and both bones were flushed using a 23-G needle on a 5 mL syringe. Bone marrow was filtered through a 70 µm filter. Incubation with 10 times diluted Pharmlyse (BD Biosciences) was performed to remove contaminating erythrocytes. The cells were washed twice in PFE buffer and were then resuspended in culture medium supplemented with M-CSF. After 24 hours, nonattached cells were washed off and after 4 days fresh M-CSF was added. After 6 days of culturing, mature macrophages were ready for PGE2 stimulation.

Lung macrophages: These were isolated from lung tissue of control mice by dissociating lung tissue and magnetic bead isolation. In brief, lungs were minced and incubated for 45 min at 37 ^{o}C in a shaking waterbath in RPMI medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Lonza), DNAse I (10 µg/ml; Roche Applied Science, Almere, The Netherlands), and collagenase I (0.66 mg/ml; Sigma-Aldrich). Purified vital lung cells were obtained by first passing the digested lung tissue through a 70 µm nylon strainer and then through a 35 µm nylon strainer. Macrophages were then isolated by positive selection using a combination of a CD11c and CD11b isolation kit (Miltenyi Biotec) and multiple rounds of magnetic sorting using an AutoMACS (Miltenyi Biotec). Positively isolated cells were cultured in DMEM medium (similar

as for RAW264.7 macrophages) with 10 ng/ml M-CSF for 24 hours at 37 °C to allow macrophages to attach to the culture dish. Nonattached cells were washed off. After 4 days of culturing with M-CSF, the macrophages were ready for PGE2 stimulation.

PGE2-stimulation: Mature macrophages were cultured for 48 hours with nothing (M_0) or 10 µM PGE2 (M_{PGE2}) and their phenotype was checked by flow cytometry by staining for CD68-PerCPCy5.5, MHC-II-APC/Cy7 and CD206-A647 (all Biolegend). M_{PGE2} macrophages were found to have higher expression of mannose receptors and no upregulation of MHC-II expression as compared to unstimulated macrophages. Macrophages were washed twice with medium to remove PGE2 before adoptive transfer.

Lung digestion for flow cytometric analysis

The left lung was minced and incubated as described for the isolation of alveolar macrophages. Single cell suspensions were incubated with 10 times diluted Pharmlyse (BD Biosciences, Breda, The Netherlands) to remove contaminating erythrocytes. Cells were centrifuged through 70 µm strainer caps and counted using a Casy cell counter (Roche Innovatis AG). Cells were subsequently used for flow cytometry.

Flow cytometric analysis of lung cell suspensions

The single lung cell suspensions were stained for T-cell and macrophage subsets using a mix of antibodies for flow cytometry.

T lymphocytes: Frequencies of effector T lymphocytes (CD3+CD4+CD25+Foxp3−) and regulatory T lymphocytes (CD3+CD4+CD25+Foxp3+) were examined using anti-CD3-APC/Cy7 (Biolegend, Fell, Germany), anti-CD4-PE/Cy7 (Biolegend), anti-CD25-PE (Biolegend), and anti-Foxp3-FITC (eBioscience, Vienna, Austria). An appropriate isotype control was used for the Foxp3 staining (rat IgG2a-FITC, eBioscience). Examples or our gating strategy can be found in supplemental figure S1.

Approximately 10^6 cells were incubated with the antibody mix including 1% normal mouse serum for 30 minutes on ice, protected from light. After washing cells with PBS supplemented with 2% FCS and 5 mM EDTA (PFE buffer), the cells were fixed and permeabilized for 30 minutes using a fixation and permeabilization buffer (eBioscience). Then cells were washed with permeabilization buffer and incubated with anti-Foxp3 including 1% normal mouse serum for 30 minutes. Subsequently, the cells were washed with permeabilization buffer, resuspended in PFE buffer and kept in the dark on ice until flow cytometric analysis.

Macrophages: Frequencies of macrophages (autofluorescent+F4/80+) and their expression of MHCII and CD206 were examined by using autofluorescence in the FITC channel, anti-F4/80-Pacific Blue (Biolegend), anti-CD206-PE (Biolegend), and anti-MHC class II-Alexa Fluor 700 (Biolegend). Examples or our gating strategy can be found in supplemental figure S2.

Approximately 10^6 cells were incubated with the appropriate antibody mix including 1% normal mouse serum for 30 minutes on ice, protected from light. After washing the cells twice with PFE buffer, cells were resuspended in FACS lysing solution (BD Biosciences) and incubated for at least 30 minutes on ice. Subsequently, cells were washed with PFE buffer and afterwards the cells were kept in the dark on ice until flow cytometric analysis.

The fluorescent staining of the cells was measured on a LSR-II flow cytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, USA).

Histology

IL-10+ macrophages were determined in 3 µm zinc-paraffin sections using standard immunohistochemical procedures. A general macrophage marker, Mac3 (anti-Mac3, BD Biosciences) was used in combination with IL-10 (Hycult Biotech, Uden, The Netherlands). Double-positive cells were counted manually in parenchymal lung tissue. Numbers were corrected for the total area of lung tissue section as measured by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, USA).

Effects of coculture of dendritic cells and macrophages on dendritic cell function

Isolation and culture of dendritic cells: Isolation and culturing protocol of bone marrow dendritic cells was similar as described above for bone marrow macrophages, except for culture medium. Bone marrow dendritic cells were cultured for 6 days in RPMI medium (Lonza) supplemented with 10% FCS (Invitrogen), 10 ng/ml GM-CSF (Peprotech, Hamburg, Germany), 0.05 mM 2-mercaptoethanol (Gibco®), 2 mM L-glutamine (Gibco®), 1 mM sodium pyruvate (Gibco®), 11 mg/l gentamicine (Gibco®) and 1% penicillin-streptomycin (Gibco®).

Coculture of dendritic cells and macrophages: Mature bone marrow-derived dendritic cells were cocultured with unstimulated lung macrophages (M_0) or PGE2-treated lung macrophages (M_{PGE2}) for 24 hours in DMEM medium (similar as for RAW264.7 macrophages). Macrophages were precultured for 48 h with 10 µM PGE2 or nothing and

thoroughly washed with medium before addition of dendritic cells. During coculture, 100 µl/mL ovalbumin was added to prepare the dendritic cells for coculture with T lymphocytes from DO11.10 T cell TCR-transgenic mice. Control coculture wells did not receive ovalbumin. Before and after coculturing, the phenotype of both macrophages and dendritic cells was checked by flow cytometry by staining with anti-MHC-II-A700, anti-CD206-A647, anti-F4/80-PB, anti-CD80-PE, anti-CD86-PE/Cy7, anti-CD40-FITC and anti-CD11c-APC/Cy7 (all Biolegend).

Isolation of T lymphocytes from DO11.10 T cell TCR-transgenic mice: T lymphocytes were isolated from the spleens of female DO11.10 T cell TCR-transgenic mice (n=6). In brief, spleen tissue was passed through a 70-µm nylon strainer (BD Biosciences) to obtain single cell suspensions. These were incubated with 10 times diluted Pharm-lyse (BD Biosciences) to remove contaminating erythrocytes. Cells were centrifuged through 70 µm strainer caps and counted using a Casy cell counter (Roche Innovatis AG). T lymphocytes were then isolated by positive selection using a CD4 isolation kit (Miltenyi Biotec) and multiple rounds of magnetic sorting using an AutoMACS (Miltenyi Biotec).

Coculture of dendritic cells and T lymphocytes from DO11.10 T cell TCR-transgenic mice: Isolated DO11.10 CD4+ T lymphocytes were resuspended in RPMI medium (Lonza) supplemented with 10% FCS (Invitrogen), 0.05 mM 2-mercaptoethanol (Gibco®), 2 mM L-glutamine (Gibco®) and 11 mg/l gentamicine (Gibco®) and seeded into a round-bottom 96-wells plate (Greiner bio-one, Alphen aan den Rijn, the Netherlands) at 100,000 cells/well. Cocultured dendritic cells were washed off the macrophage cultures with medium and were added at 100,000 cells/well for coincubation with CD4+ T lymphocytes for 48 hours.

T lymphocyte proliferation assay: 50 µl of T lymphocyte medium was taken from each well after 48 hours of coincubation with dendritic cells. This was snap-frozen and stored at -80°C for cytokine analysis. This 50 µl of medium was replaced with 50 µl fresh medium containing 1 µCi ^{3}H-thymidine (PerkinElmer, Zaventem, Belgium). After 18 hours, ^{3}H-thymidine-incorporation was measured using a scintillation counter. A flow chart of the macrophage-dendritic coculture experiment is depicted in figure 2.

Cytokine assay

Levels of IL4, IL5, IL6, IL10, IL12, IL13, IL17, TNFα and IFNγ were measured in T lymphocyte medium by multiplex ELISA and a Luminex analyzer, according to the manufacturer's recommendations (eBioscience).

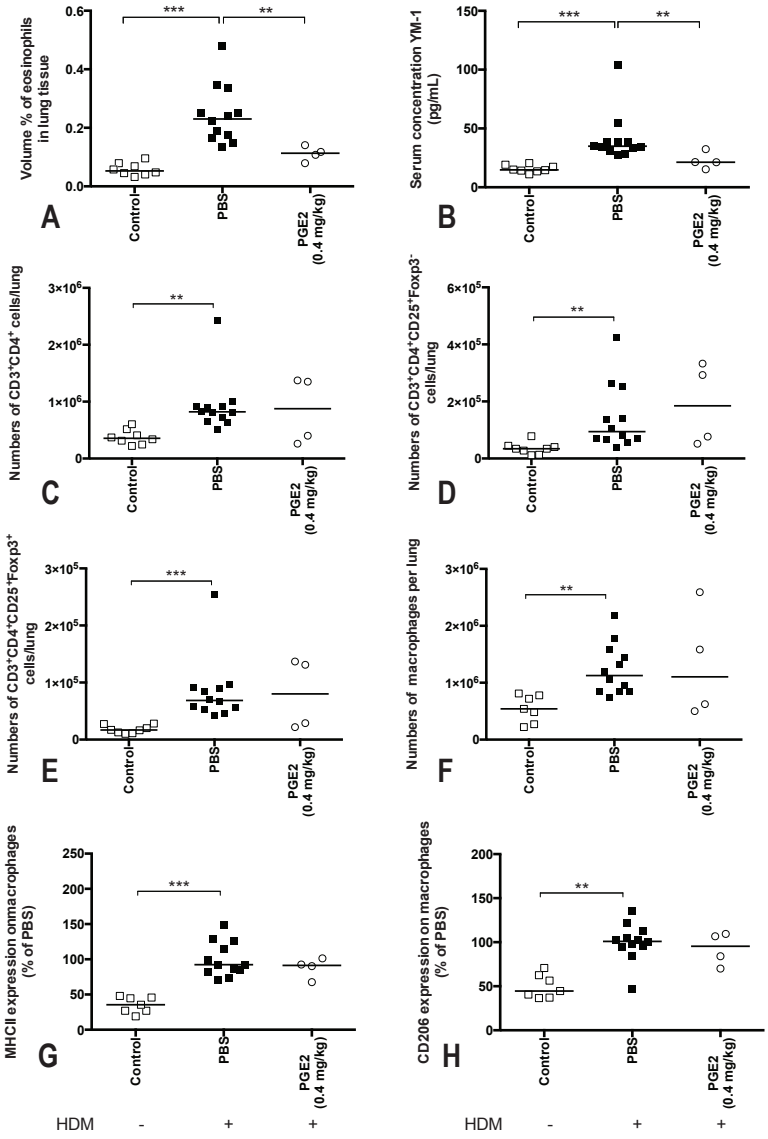

Figure 2: Mice exposed to house dust mite (HDM) and treated with phosphate-buffered saline (PBS) during HDM exposure have significantly more infiltrating eosinophils in lung tissue (**panel A**), higher levels of YM1 in serum (**panel B**), more CD4+ T lymphocytes (**panel C**), more activated CD4+ T lymphocytes (**panel D**), more regulatory T lymphocytes (**panel E**), more macrophages (**panel F**), higher expression of MHCII (**panel G**) and CD206 (**panel H**) on macrophages in lung tissue than healthy control mice. Treatment with 0.4 mg/kg PGE2 resulted in significantly lower eosinophil infiltration in lung tissue and lower levels of YM1 as compared to PBS-treated animals, but had no effect on the infiltrating T lymphocyte subsets and the number of macrophages in lung tissue or the expression of MHCII and CD206 on these macrophages. **p<0.01, ***p<0.001 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing control vs. PBS and PBS vs. PGE2.

Statistics

Data are represented as mean ± standard error of the mean. To determine the normality of the data the Shapiro-Wilk normality test was used. Data were log-transformed to fit a normal distribution when not normally distributed. In case of normal distribution, differences between groups were tested using a one-way ANOVA followed by Holm-Sidak's multiple comparisons test. In case of nonnormal distribution, differences between groups were tested using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. P-values <0.05 were considered to be statistically significant.

Results and discussion

In this study we boosted the anti-inflammatory function of macrophages by stimulating IL10 expression. Our previous studies showed that IL10-expressing macrophages disappear in lung tissue of human asthmatics and in mouse models of allergic and nonallergic lung inflammation, while macrophages associated with IL4/IL13 stimulation increase [1, 4, 17 and manuscript submitted]. We therefore incubated RAW264.7 macrophages with a number of substances that have been reported to stimulate IL10 expression in macrophages, namely adenosine, PGE2 and lipopolysaccharide (LPS) [18–21]. Of these three, PGE2 was the only one that could significantly stimulate IL10 expression in RAW264.7 macrophages, leading to a five-fold increase in the number of IL10-positive macrophages (figure 1A). PGE2 is a well-known anti-asthmatic compound that has been shown to prevent allergen-induced bronchoconstriction and inhibit airway hyperresponsiveness and inflammation [22]. These anti-asthmatic effects were shown to be mediated through E prostanoid receptors 2 and 4 (EP2 and EP4) on T lymphocytes, monocytes/macrophages, mast cells, and bronchial smooth muscle cells [23–28]. This wide range of target cells of course presented us with a challenge trying to show anti-inflammatory effects of PGE2 on macrophages specifically. Therefore, we also studied a more macrophage-specific preparation of PGE2, namely PGE2 coupled to mannosylated human serum albumin (PMH). We have shown before that mannosylated albumin is taken up specifically by macrophages expressing the mannose receptor (CD206) [29]. As macrophages in asthmatic lungs are characterized by high expression of CD206 [1, 17], we coupled PGE2 to mannosylated albumin for macrophage-specific uptake and possibly macrophage-specific effects. Treating macrophages with 100 µg/ml PMH induced similar high numbers of IL10 expressing macrophages as compared to an equimolar amount of free PGE2 (figure 1B). When lowering the dose of either PMH or free PGE2 tenfold or a hundredfold, we found a dose-dependent reduction of the number of IL10-expressing macrophages. Macro-

phages treated with mannosylated HSA in similar concentrations as PMH were not stimulated to express more IL10 as compared to untreated control macrophages.

Having found that both free PGE2 and PMH could stimulate IL10 expression in macrophages *in vitro*, we treated mice with three different concentrations of each compound during the induction of allergic lung inflammation to investigate whether they could dampen inflammation. Exposure to HDM induced significant higher numbers of eosinophils, (activated) CD4+ T lymphocytes, regulatory T lymphocytes and higher levels of YM1 in lung tissue as compared to control mice (figure 2A-E). Treatment with free PGE2 led to a clear and dose-dependent reduction of eosinophils and YM1 in lung tissue, with the highest dose being the most effective. The results presented in figure 2 show the effects of the highest PGE2 dose of 0.4 mg/kg, the results of the lower doses can be found in supplemental figures S2A-B. There was no effect of this PGE2 dose on numbers of the different T lymphocyte subsets. HDM exposure also led to significantly more macrophages in lung tissue as compared to control (figure 2F) and these HDM-exposed macrophages expressed more CD206 and MHCII than macrophages from control lung tissue (figures 2G-H). Treatment with 0.4 mg/kg PGE2 did not affect either numbers of lung macrophages or expressions of CD206 or MHCII on these macrophages.

Thus, we could confirm that free PGE2 instilled in the lungs had anti-inflammatory potential as was shown in many studies before, but whether this was due to a specific effect on macrophages was unclear. Our macrophage-specific construct of PGE2 coupled to mannosylated human serum albumin (7.4 mg/kg PMH) induced IL10 expression significantly in macrophages *in vitro*, but failed to have any anti-inflammatory effects *in vivo* (figures 3A-H). There are several possible explanations for this phenomenon. The use of mannosylated albumin directs the construct towards uptake through mannose receptors [29], degradation of the construct in lysosomes and potentially to the release of free PGE2. The receptors for PGE2 are of course on the outside of the cell and the effectiveness of this approach relies on the PGE2 being released outside the cell again. Our *in vitro* experiments showed this does work and PGE2 coupled to mannosylated albumin can be as effective as free PGE2. However, *in vivo* distribution and elimination effects may have impeded its effects. For instance, mature alveolar macrophages have a much higher expression of mannose receptors than interstitial or monocyte-derived macrophages [30] and may have scavenged most of the construct due to their high receptor density and their prime location on the treatment side in the lungs (the construct was instilled intranasally). This is of relevance because studies from Zaslona *et al.* and Gundra *et al.* have shown that mature alveolar macrophages protect against allergic lung inflammation whereas, newly derived macrophages from

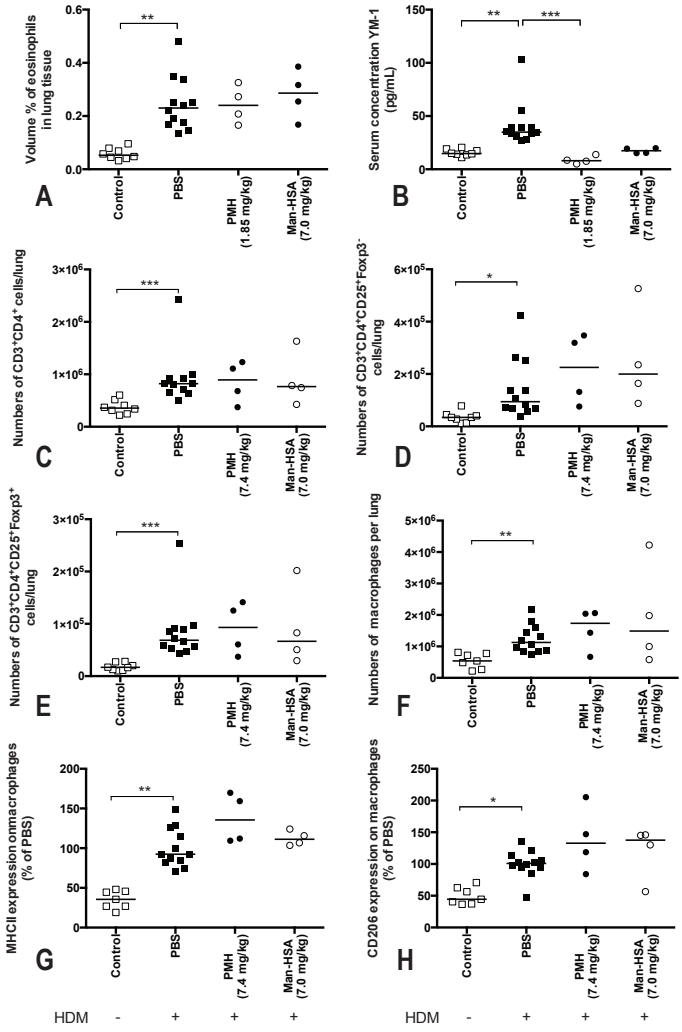

Figure 3: Mice exposed to house dust mite (HDM) and treated with phosphate-buffered saline (PBS) during HDM exposure have significantly more infiltrating eosinophils in lung tissue (**panel A**), higher levels of YM1 in serum (**panel B**), more CD4+ T lymphocytes (**panel C**), more activated CD4+ T lymphocytes (**panel D**), more regulatory T lymphocytes (**panel E**), more macrophages (**panel F**), higher expression of MHCII (**panel G**) and CD206 (**panel H**) on macrophages in lung tissue than healthy control mice. Treatment with 7.4 mg/kg PGE2 coupled to mannosylated human serum albumin (PMH, equimolar to 0.4 mg/kg PGE2) did not affect eosinophil, CD4+ T lymphocytes infiltration, number of macrophages in lung tissue or the expression of MHCII and CD206 on these macrophages in lung tissue. Treatment with 7.4 mg/kg PMH did result in lower levels of YM1 in serum as compared to PBS-treated animals, but this was due to an effect of treatment with empty carrier mannosylated human serum albumin (Man-HSA), as treatment with an equimolar amount of 7.0 mg/kg Man-HSA had the same effect. Treatment with Man-HSA did not affect infiltration of eosinophils, T lymphocytes, number of macrophages in lung tissue or the expression of MHCII and CD206 on these macrophages. *P<0.05, **p<0.01, ***p<0.001 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing control vs. PBS, PBS vs. Man-HSA, and Man-HSA vs. PMH.

either proliferation or recruited monocytes may be promoting development of inflammation [10, 31]. Therefore, the mannose receptor-directed version of PGE2 may have been scavenged by the cells that do not need to be modified.

The only clear effect the PGE2-construct had, was a reduction in YM1 levels in serum. YM1 is highly produced by IL4/IL13-stimulated macrophages in lung tissue and we have found that levels in serum reflect lung tissue well in HDM-induced lung inflammation (data not shown). As mannosylated albumin had the same effect with or without PGE2 being present, this appears to be the result of some interaction of the either the mannosylated construct with YM1 or the mannose receptor with YM1 production. To date we have not been able to find any evidence to explain this observation.

As our macrophage-specific PGE2 formulation was not effective, we treated macrophage *ex vivo* with PGE2 to induce their anti-inflammatory phenotype and adoptively transferred them into the lungs during the induction of allergic lung inflammation with HDM. This also gave us the opportunity to test whether macrophages with a hematopoietic origin would have a different effect compared to lung macrophages with an embryonic origin. We did not observe any differences between the effects of lung macrophages or bone marrow-derived macrophages and we therefore combined these groups in figure 4. Adoptive transfer of either *unstimulated* lung or bone marrow-derived macrophages had very little effect on development of allergic lung inflammation. There was a trend towards more eosinophils infiltrating lung tissue of HDM-exposed mice that were treated with unstimulated macrophages as compared to untreated HDM-exposed mice (figure 4A, p=0.06). Treatment with PGE2-treated macrophages did affect development of HDM-induced inflammation. Overall less inflammation was seen with less infiltrating eosinophils (trend, p=0.06, figure 4A) and less infiltrating CD4+ T lymphocytes (figure 4C), with both activated T lymphocytes and regulatory T lymphocytes being down (figure 4D-E) as compared to treatment with unstimulated macrophages. In addition, we found a trend towards less macrophages being present (p=0.08, figure 5A) and a trend towards higher numbers of IL10+ macrophages being present in lung tissue as compared to treatment with unstimulated macrophages (p=0.15, figure 5B). Other macrophage phenotypes (i.e. expression of MHCII or CD206, figure 5C-D) had not changed as compared to treatment with unstimulated macrophages. Thus, this approach inhibited more HDM-induced effects than treatment with free PGE2 and this may have been caused by the higher levels of PGE2 the adoptively transferred macrophages were exposed to *in vitro* or the fact that multiple cell types in the lung are affected by free PGE2, which may have counterbalanced some of the macrophage-specific effects. These findings indicate that the lower numbers of the IL10-producing macrophages we have found

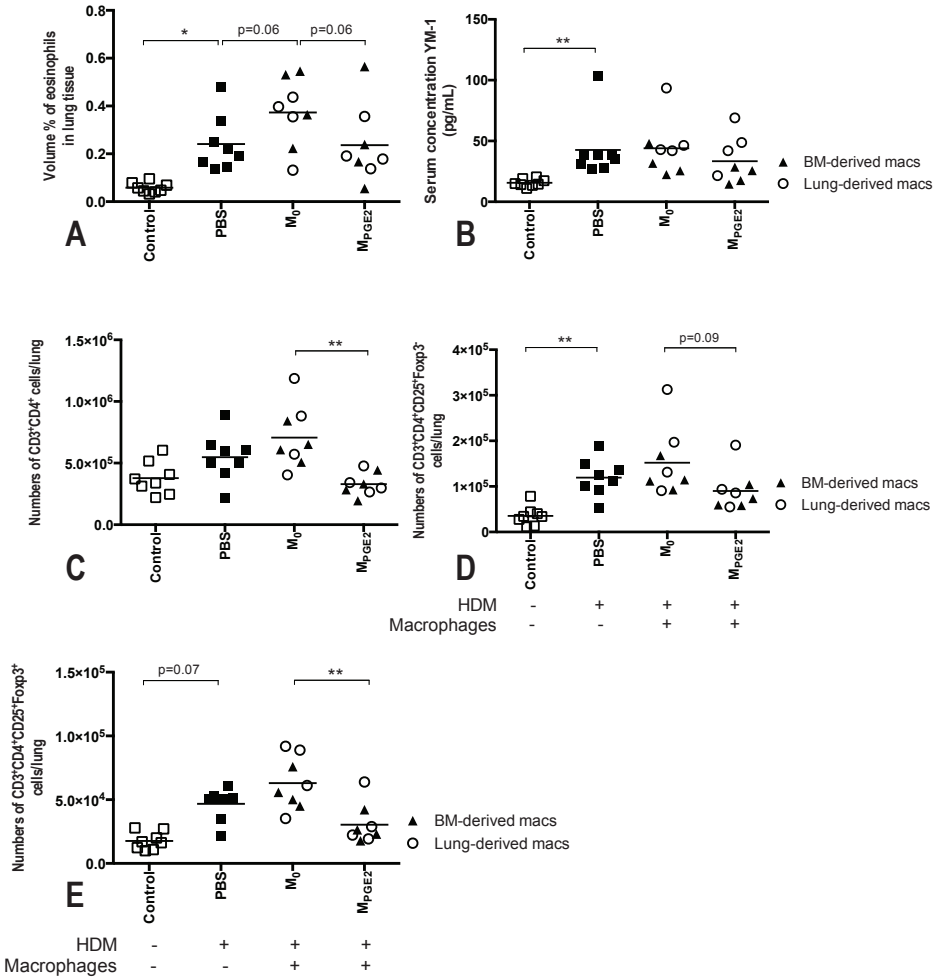

Figure 4: Mice exposed to house dust mite (HDM) and treated with phosphate-buffered saline (PBS) during HDM exposure have significantly more infiltrating eosinophils in lung tissue (**panel A**), higher levels of YM1 in serum (**panel B**), equal numbers of CD4+ T lymphocytes (**panel C**), more activated CD4+ T lymphocytes (**panel D**), and a trend towards more regulatory T lymphocytes (**panel E**) in lung tissue than healthy control mice. Adoptive transfer of untreated (M_0) lung macrophages (**open circles**) or untreated bone marrow (BM)-derived macrophages (**closed triangles**) did not affect these parameters except for a trend towards more eosinophils in lung tissue as compared to PBS-treated animals. Adoptive transfer of PGE2-treated lung or BM-derived macrophages (M_{PGE2}) resulted in a trend towards less eosinophil and effector T lymphocyte infiltration into lung tissue and significantly less CD4+ and regulatory T lymphocytes infiltration into lung tissue as compared to mice that received unstimulated macrophages. There was no statistical difference between adoptively transferring lung macrophages or BM-derived macrophages. *P<0.05, **p<0.01 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing control vs. PBS, PBS vs. M_0, and M_0 vs. M_{PGE2}.

asthma patients and in mouse models of asthma are important in the development of allergic inflammation because re-introducing these macrophages into lung tissue

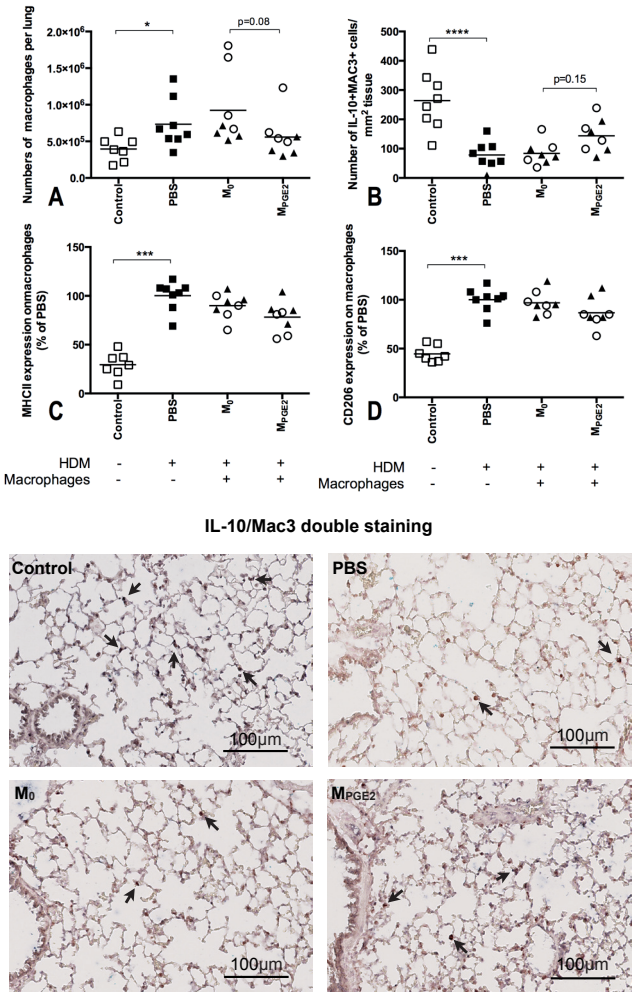

Figure 5: Mice exposed to house dust mite (HDM) and treated with phosphate-buffered saline (PBS) during HDM exposure have significantly more total macrophages in lung tissue (**panel A**), less IL10+ macrophages (**panel B**), and higher expression of MHCII (**panel C**) and CD206 (**panel D**) on these macrophages than healthy control mice. Adoptive transfer of untreated (M_0) lung macrophages (**open circles**) or untreated bone marrow (BM)-derived macrophages (**closed triangles**) did not affect these parameters compared to PBS-treated animals. Adoptive transfer of PGE2-treated lung or BM-derived macrophages (M_{PGE2}) resulted in a trend towards less total macrophages and more IL10+ macrophages in lung tissue as compared to mice that received unstimulated macrophages. Expressions of MHCII and CD206 were not affected by PGE2-treated macrophages and there was no statistical difference between adoptively transferring lung macrophages or BM-derived macrophages.

Representative photos of the IL10/Mac3 double stainings are shown in **panels E-H**. Nuclear counter staining was omitted to maximize visibility of double-positive cells (magnification 200x). Lung sections were stained for IL10 (blue) and a general macrophage marker Mac3 (red) to specifically identify IL10+ macrophages. The arrowheads indicate some examples of double-positive cells. Double positive cells were counted and related to total lung tissue surface as depicted in **panel B**. *P<0.05, ***p<0.001 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing control vs. PBS, PBS vs. M_0, and M_0 vs. M_{PGE2}.

has obvious beneficial effects [4, 17]. Whether these PGE2-treated macrophages are also effective in asthma that already has fully developed is an important question that needs further studies. If so, this could open up a whole new therapeutic perspective for the treatment of asthma.

An open question is the mechanism by which PGE2-treated macrophages inhibit HDM-induced lung inflammation. This could be due to increased expression of IL10 by macrophages, as we found a trend towards more IL10-expressing macrophages in lung tissue of the mice receiving PGE2-treated macrophages. The group of Holt described another possible mechanism many years ago. They showed that alveolar macrophages can both downregulate the antigen presenting cell functions of dendritic cells and directly inhibit T lymphocyte proliferation themselves [13, 32, 33]. As we found that the inhibition of HDM-induced lung inflammation by PGE2-treated macrophages was most pronounced for the infiltration of CD4+ T lymphocytes, we subsequently investigated whether this effect was mediated through modulation of dendritic cell function by PGE2-treated macrophages. In order to do this we incubated unstimulated or PGE2-treated lung macrophages with bone marrow-derived dendritic cells with or without ovalbumin. After 24 hours of coculture the dendritic cells were then cocultured with ovalbumin-specific CD4+ T lymphocytes to study effects on T lymphocyte proliferation and cytokine production. Dendritic cells in the presence of ovalbumin and unstimulated macrophages could effectively induce T lymphocytes proliferation with no particular direction in the response as Th1, Th2, and Th17-related cytokines were all produced more than when dendritic cells were not preincubated with ovalbumin and unstimulated macrophages (table 1). Preincubating dendritic cells with ovalbumin and PGE2-treated macrophages did not make any difference for T lymphocyte proliferation and cytokine production as compared to preincubation with unstimulated macrophages. Thus, we could not find evidence for this hypothesis. It is therefore likely these anti-inflammatory macrophages have a local effect either through inhibiting T lymphocyte proliferation in lung tissue or through other anti-inflammatory effects. The Peters-Golden group recently published an interesting suggestion with respect to this latter option. They showed that anti-inflammatory effects of PGE2-treated macrophages can be mediated through increased transcellular delivery of vesicular SOCS (suppressor of cytokine signaling) proteins [34]. Elucidating these pathways further may give valuable information on how to stimulate anti-inflammatory behavior in macrophages without the help of PGE2. This is of particular interest because the development of PGE2 as a drug in asthma has been hindered by its propensity to induce cough [35, 36].

Table 1: Bone marrow-derived dendritic cells (DC) preincubated with untreated macrophages (M_0) and cocultured with ovalbumin-specific CD4+ T lymphocytes induced significantly more T lymphocyte proliferation and cytokine production when also exposed to ovalbumin (OVA) as compared to no OVA being present. Preincubation of DC with PGE2-treated macrophages (M_{PGE2}) did not affect this T lymphocyte proliferation and cytokine production. *P<0.05, ***p<0.001 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test comparing no-OVA vs. OVA+M_0 and OVA+M_0 vs. OVA+M_{PGE2}

	DC + T lymphocytes cocultured with no OVA (n=6)	DC (preincubated with M_0) + T lymphocytes + OVA (n=6)	DC (preincubated with M_{PGE2}) + T lymphocytes + OVA (n=4)
T lymphocyte proliferation (% of M_0 + OVA)	**20±5** ***	100±0	122±67
TNFa production (% of M_0 + OVA)	**5.2±1.2** *	100±0	148±28
IFNg production (% of M_0 + OVA)	**0.3±0.2** *	100±0	107±14
IL6 production (% of M_0 + OVA)	**0.7±0.4** *	100±0	197±65
IL12p70 production (% of M_0 + OVA)	**4.0±2.4** *	100±0	112±22
IL-4 production (% of M_0 + OVA)	**0.8±0.3** **	100±0	81±17
IL5 production (% of M_0 + OVA)	**0.3±0.2** **	100±0	74±17
IL13 production (% of M_0 + OVA)	**0.2±0.1** **	100±0	95±17
IL10 production (% of M_0 + OVA)	**0±0** *	100±0	119±15
IL17 production (% of M_0 + OVA)	**0.1±0.03** *	100±0	103±16

In conclusion, our study has shown that redirecting macrophage polarization towards an anti-inflammatory IL10-producing phenotype by using PGE2 inhibits the development of allergic lung inflammation. This beneficial effect of repolarization was independent of macrophage origin, increasing the potential of the approach for therapeutic purposes.

Acknowledgements

This work was supported by grant 3.2.10.056 from the Netherlands Asthma Foundation (BNM). In addition, we thank Pharmacy students Marriël Schelhaas and Corine Habraken for their practical contributions to this paper. BNM is an active member of COST action BM1201.

References

1 Melgert BN, ten Hacken NH, Rutgers B, Timens W, Postma DS, Hylkema MN. More alternative activation of macrophages in lungs of asthmatic patients. *J. Allergy Clin. Immunol.* 2011; 127: 831–833.

2 Bhakta NR, Woodruff PG. Human asthma phenotypes: from the clinic, to cytokines, and back again. *Immunol. Rev.* 2011; 242: 220–232.

3 Melgert BN, Oriss TB, Qi Z, Dixon-McCarthy B, Geerlings M, Hylkema MN, Ray A. Macrophages: regulators of sex differences in asthma? *Am. J. Respir. Cell Mol. Biol.* 2010; 42: 595–603.

4 Draijer C, Robbe P, Boorsma CE, Hylkema MN, Melgert BN. Characterization of macrophage phenotypes in three murine models of house-dust-mite-induced asthma. *Mediators Inflamm.* 2013; 2013: 632049.

5 Martinez FO, Helming L, Milde R, Varin A, Melgert BN, Draijer C, Thomas B, Fabbri M, Crawshaw A, Ho LP, Ten Hacken NH, Cobos Jiménez V, Kootstra NA, Hamann J, Greaves DR, Locati M, Mantovani A, Gordon S. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood* 2013; 121: e57–e69.

6 Staples KJ, Hinks TSC, Ward JA, Gunn V, Smith C, Djukanović R. Phenotypic characterization of lung macrophages in asthmatic patients: overexpression of CCL17. *J. Allergy Clin. Immunol.* 2012; 130: 1404–1412.e7.

7 Guilliams M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, Deswarte K, Malissen B, Hammad H, Lambrecht BN. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J. Exp. Med.* 2013; 210: 1977–1992.

8 Yona S, Kim K-W, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guilliams M, Misharin A, Hume DA, Perlman H, Malissen B, Zelzer E, Jung S. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 2013; 38: 79–91.

9 Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J, Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, van Rooijen N, García-Sastre A, Stanley ER, Ginhoux F, Frenette PS, Merad M. Tissue resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity.* 2013; 38: 792-804.

10 Zasłona Z, Przybranowski S, Wilke C, van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, Wilkinson JE, Moore BB, Peters-Golden M. Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma. *J. Immunol.* 2014; 193: 4245–4253.

11 Lee YG, Jeong JJ, Nyenhuis S, Berdyshev E, Chung S, Ranjan R, Karpurapu M, Deng J, Qian F, Kelly EAB, Jarjour NN, Ackerman SJ, Natarajan V, Christman JW, Park GY. Recruited Alveolar Macrophages, in Response to Airway Epithelial-derived MCP-1/CCL2, Regulate Airway Inflammation and Remodeling in Allergic Asthma. *Am. J. Respir. Cell Mol. Biol.* 2014; 52:772-784.

12 Careau E, Bissonnette EY. Adoptive transfer of alveolar macrophages abrogates bronchial hyperresponsiveness. *Am. J. Respir. Cell Mol. Biol.* 2004; 31: 22–27.

13 Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* 1993; 177: 397–407.

14 Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity* 2010; 32: 593–604.

15 Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege J-L, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 2014; 41: 14–20.

16 Martinez FO, Gordon S. The evolution of our understanding of macrophages and translation of findings toward the clinic. *Expert Rev. Clin. Immunol.* 2015; 11: 5–13.

17 Robbe P, Draijer C, Borg TR, Luinge M, Timens W, Wouters IM, Melgert BN, Hylkema MN. Distinct macrophage phenotypes in allergic and nonallergic lung inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2015; 308: L358–L367.

18 Németh ZH, Lutz CS, Csóka B, Deitch EA, Leibovich SJ, Gause WC, Tone M, Pacher P, Vizi ES, Haskó G. Adenosine Augments IL-10 Production by Macrophages through an A2B Receptor-Mediated Posttranscriptional Mechanism. *J. Immunol.* 2005; 175: 8260–8270.

19 Harizi H, Juzan M, Pitard V, Moreau J-F, Gualde N. Cyclooxygenase-2-issued prostaglandin e(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J. Immunol.* 2002; 168: 2255–2263.

20 MacKenzie KF, Clark K, Naqvi S, McGuire VA, Nöehren G, Kristariyanto Y, van den Bosch M, Mudaliar M, McCarthy PC, Pattison MJ, Pedrioli PGA, Barton GJ, Toth R, Prescott A, Arthur JSC. PGE(2) induces macrophage IL-10 production and a regulatory-like phenotype via a protein kinase A-SIK-CRTC3 pathway. *J. Immunol.* 2013; 190: 565–577.

21 van den Bosch MWM, Palsson-Mcdermott E, Johnson DS, O'Neill LAJ. LPS induces the degradation of programmed cell death protein 4 (PDCD4) to release Twist2, activating c-Maf transcription to promote interleukin-10 production. *J. Biol. Chem.* 2014; 289: 22980–22990.

22 Chung KF. Evaluation of selective prostaglandin E2 (PGE2) receptor agonists as therapeutic agents for the treatment of asthma. *Sci. STKE Signal Transduct. Knowl. Environ.* 2005; 2005: pe47.

23 Birrell MA, Maher SA, Dekkak B, Jones V, Wong S, Brook P, Belvisi MG. Anti-inflammatory effects of PGE2 in the lung: role of the EP4 receptor subtype. *Thorax* 2015; 70: 740–747.

24 Serra-Pages M, Torres R, Plaza J, Herrerias A, Costa-Farré C, Marco A, Jiménez M, Maurer M, Picado C, de Mora F. Activation of the Prostaglandin E2 receptor EP2 prevents house dust mite-induced airway hyperresponsiveness and inflammation by restraining mast cells' activity. *Clin. Exp. Allergy.* 2015; 45: 1590–1600.

25 Serra-Pages M, Olivera A, Torres R, Picado C, de Mora F, Rivera J. E-prostanoid 2 receptors dampen mast cell degranulation via cAMP/PKA-mediated suppression of IgE-dependent signaling. *J. Leukoc. Biol.* 2012; 92: 1155–1165.

26 Benyahia C, Gomez I, Kanyinda L, Boukais K, Danel C, Leséche G, Longrois D, Norel X. PGE(2) receptor (EP(4)) agonists: potent dilators of human bronchi and future asthma therapy? *Pulm. Pharmacol. Ther.* 2012; 25: 115–118.

27 Zasłona Z, Okunishi K, Bourdonnay E, Domingo-Gonzalez R, Moore BB, Lukacs NW, Aronoff DM, Peters-Golden M. Prostaglandin E_2 suppresses allergic sensitization and lung inflammation by targeting the E prostanoid 2 receptor on T cells. *J. Allergy Clin. Immunol.* 2014; 133: 379–387.

28 Säfholm J, Manson ML, Bood J, Delin I, Orre A-C, Bergman P, Al-Ameri M, Dahlén S-E, Adner M. Prostaglandin E2 inhibits mast cell-dependent bronchoconstriction in human small airways through the E prostanoid subtype 2 receptor. *J. Allergy Clin. Immunol.* 2015; 136: 1232–1239.e1.

29 Melgert BN, Olinga P, Van Der Laan JM, Weert B, Cho J, Schuppan D, Groothuis GM, Meijer DK, Poelstra K. Targeting dexamethasone to Kupffer cells: effects on liver inflammation and fibrosis in rats. *Hepatol.* 2001; 34: 719–728.

30 Ezekowitz RA, Stahl PD. The structure and function of vertebrate mannose lectin-like proteins. *J. Cell Sci. Suppl.* 1988; 9: 121–133.

31 Gundra UM, Girgis NM, Ruckerl D, Jenkins S, Ward LN, Kurtz ZD, Wiens KE, Tang MS, Basu-Roy U, Mansukhani A, Allen JE, Loke P 'ng. Alternatively activated macrophages derived from monocytes and tissue macrophages are phenotypically and functionally distinct. *Blood* 2014; 123: e110–e122.

32 Upham JW, Strickland DH, Bilyk N, Robinson BW, Holt PG. Alveolar macrophages from humans and rodents selectively inhibit T-cell proliferation but permit T-cell activation and cytokine secretion. *Immunology* 1995; 84: 142–147.

33 Upham JW, Strickland DH, Robinson BW, Holt PG. Selective inhibition of T cell proliferation but not expression of effector function by human alveolar macrophages. *Thorax* 1997; 52: 786–795.

34 Bourdonnay E, Zasłona Z, Penke LRK, Speth JM, Schneider DJ, Przybranowski S, Swanson JA, Mancuso P, Freeman CM, Curtis JL, Peters-Golden M. Transcellular delivery of vesicular SOCS proteins from macrophages to epithelial cells blunts inflammatory signaling. *J. Exp. Med.* 2015; 212: 729–742.

35 Coleridge HM, Coleridge JC, Ginzel KH, Baker DG, Banzett RB, Morrison MA. Stimulation of "irritant" receptors and afferent C-fibres in the lungs by prostaglandins. *Nature* 1976; 264: 451–453.

36 Maher SA, Belvisi MG. Prostanoids and the cough reflex. *Lung* 2010; 188: S9–S12.

Supplemental data

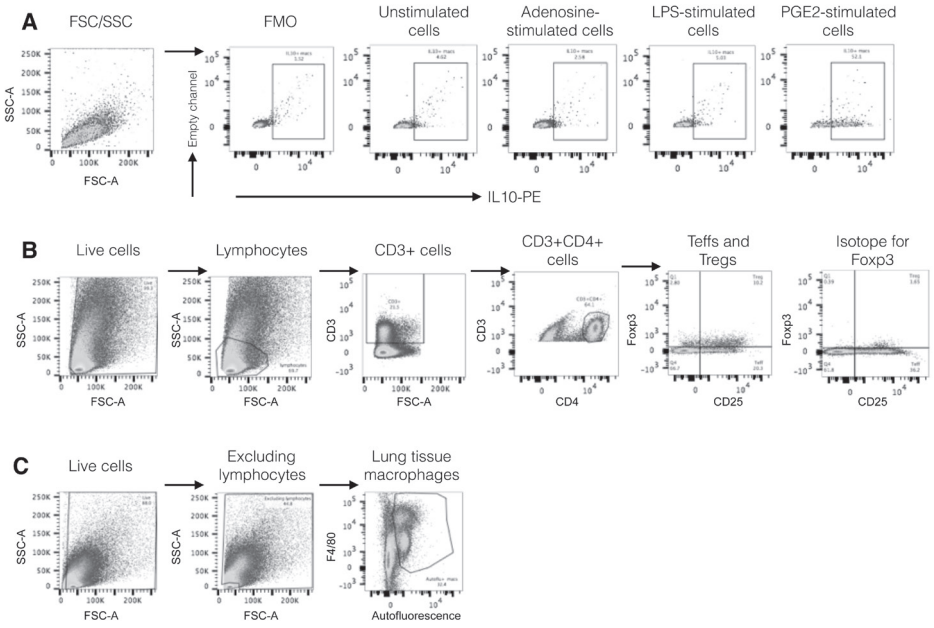

Figure S1: (A) Gating strategy for IL10-positive RAW264.7 macrophages. **(B)** Gating strategy of CD4+ T lymphocytes, effector T lymphocytes (Teffs) and regulatory T lymphocytes (Tregs) in lung tissue. **(C)** Gating strategy for lung tissue macrophages.

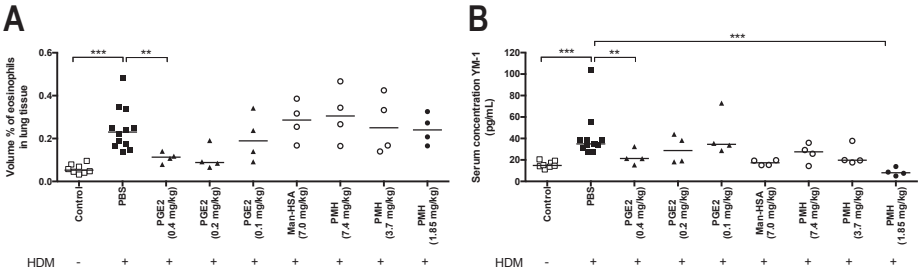

Figure S2: Mice exposed to house dust mite (HDM) and treated with phosphate-buffered saline (PBS) during HDM exposure have significantly more infiltrating eosinophils (panel A) and higher levels of YM1 in serum (panel B) than healthy control mice. Treatment with increasing doses of PGE2 resulted in significantly lower eosinophil infiltration in lung tissue and lower levels of YM1 as compared to PBS-treated animals. Treatment with equimolar increasing doses of PGE2 coupled to mannosylated human serum albumin (PMH) did not affect numbers of eosinophils in lung tissue, but did result in significantly lower levels of YM1 as compared to PBS-treated animals. This effect was caused by mannosylated human serum albumin (Man-HSA) and not by PGE2 because man-HSA had the same effect as PMH. **p<0.01, ***p<0.001 using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test.